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Production of Tumor Necrosis Factor-alpha, Interleukin 1-beta, Interleukin 2, and Interleukin 6 by rat leukocyte subpopulations after exposure to Substance P

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Abstract

The interaction between components of the nervous system and multiple target cells in the cutaneous immune system has been receiving increasing attention. Recently, the involvement of neuropeptides has been demonstrated to play an important role in the inflammatory cascade. Neuropeptides such as Substance P are released by cutaneous neurons and modulate the function of immunocompetent and inflammatory cells as well as epithelial and endothelial cells. Substance P has been shown to function as a mediator for cell proliferation, cytokine production, and as an upregulator of various cell surface receptors. In this study, we show the effect of Substance P on the production of Tumor Necrosis Factor-alpha, Interleukin 1-beta, Interleukin 2, and Interleukin 6 by T-lymphocytes, macrophages and neutrophils. These data demonstrate that pathophysiological levels of Substance P induce production of cytokines in all three cell populations tested. Interestingly, T-cells demonstrated the highest percentage of cells expressing all four cytokines. In contrast, macrophages and neutrophils produced the highest absolute levels of cytokines. The elucidation of mediating mechanisms of Substance P activation of leukocytes is crucial to the understanding of the cutaneous inflammatory cascade and involvement of the peripheral nervous system on the immune system. These findings suggest that Substance P participates in the complex network of mediators that regulate cutaneous inflammation and potentially the rate of wound healing.

Keywords: Substance P; Cytokines; Leukocyte; Flow cytometry; Intracellular

1. Introduction

Substance P (SP) has been extensively studied and thus is considered a mainstream neuropeptide player, one of more than fifty known neuroactive molecules. The understanding of SP has evolved since the original concept as a pain transmitter in the dorsal horn. Seventy years of research have lead to the current understanding of the diverse functions of this neuropeptide. Although extensively studied, there still remain many unanswered questions germane to the different roles played by SP and its involvement in a variety of physiological systems, especially with regards to tissue repair resulting from trauma.

Our interest centers about SP functioning as an integral part of both nervous and immune system interaction. We are desirous of addressing how the peripheral nervous system (PNS), acting through neuropeptides such as SP, not only relays sensory information to the central nervous system (CNS) but also plays an effector role in the inflammatory, proliferative, and reparative processes after injury. There is evidence suggesting neurogenic stimuli affecting cellular events involved in inflammation, proliferation, and the extracellular matrix, as well as cytokine and growth factor synthesis (Payan, 1989). It is also important to note that neuronal cells are not the only cell type that produces SP. SP is

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expressed by immune cells as well. Published reports show that morphine up-regulates the expression of SP produced by human monocytes and lymphocytes in culture (Li et al., 2000). Lai et al., have demonstrated SP mRNA expression in human white blood cells (Lai et al., 2002). Furthermore, It has been shown that HIV-infected patients have elevated plasma SP levels as compared to uninfected subjects (Ho et al., 2002).

Currently, there exist controversy regarding the effect of SP induction of activation of inflammatory cells and subsequent cytokine secretion. This controversy was initiated after published data indicated monocytes activated by lipopolysaccharide (LPS) are preferentially affected by SP (Laurenzi et al., 1990). Re-examination of published reports regarding this issue have lead to the belief that previous reports describing the direct effect of SP on cytokine production probably arose from low endotoxin contamination (Brain, 1997). A recent study demonstrated that SP induced cytokine production is mediated via the SP receptor as evidenced by the blocking of the SP receptor and subsequent abolishment of cytokine synthesis (Veronesi et al., 1999). Similar findings have been observed in rodent cells (Kawamura et al., 1998).

Tumor Necrosis Factor-alpha (TNF-α), Interleukin 1-beta (IL-1β), Interleukin 2 (IL-2), and Interleukin 6 (IL-6) are frequently encountered proinflammatory cytokines. These cytokines are involved in a variety of immunological functions as well as interaction with a variety of target cells. IL-6 has been shown to promote terminal differentiation of proliferating B-cells to plasma cells, stimulation of antibody secretion, and inducing synthesis of acute-phase proteins. IL-6 is secreted primarily by monocytes and macrophages. This cytokine is always found in increased levels at sites of inflammation and is likely very important in a number of to date poorly understood inflammatory regulation schemes. IL-2 is secreted predominantly by T-cells and has been shown to have proliferative effects as well as enhancing activity of natural killer (NK) cells. IL-1β is an important part of the inflammatory response. An inflammation associated function of IL-1β is its action on CNS endothelial cell receptors involving the resetting of the thermoregulatory centre, thus increasing the core body temperature and manifested as fever. Furthermore, IL-1β co-stimulates activation of T-cells, promotes maturation of B-cells, enhances NK activity, increases adhesion molecules expression, and acts as a chemotactic attractant. Cells that have been shown to secrete IL-1β are monocytes, macrophages, B-cells, dendritic cells, endothelial cells, neutrophils, and hepatocytes. TNF-α is secreted by macrophages and mast cells and has been shown to exhibit cytotoxic properties to tumor cells and germane to our interest, been associated with chronic inflammation. TNF-α has been associated with the induction of apoptosis through NF-kB, a protein reported to be a major immune response-modifying cytokine produced primarily by activated macrophages. TNF-α induces expression of other autocrine growth factors and increases cellular responsiveness to growth factors, inducing signaling pathways that lead to proliferation. TNF-α acts synergistically with other cytokine and growth factors on some cell types. Like other growth factors, TNF-α has been associated with wound healing. Both TNF-α and IL-1β have been proposed to induce activation of fibroblasts and keratinocytes leading to secretion of nerve growth factor (NGF) associated with wound healing. This last function is critical since it has been shown that NGF is capable of directly increasing the synthesis of SP in dorsal root ganglion cells (Shaffer et al., 1998). Further elucidation of the role of SP as an activator of cytokine production is of critical importance, especially in inflammation and tissue repair. For such studies, rodents have been the preferred species. This study seeks to understand the effects of SP on cytokine synthesis in a variety of rat cell populations affording some insight into the effect of SP on infiltrating inflammatory cells and its respective contribution to wound healing.

For these reasons, finding cross-reactivity of well-characterized human antibodies would be a welcome tool in research laboratories. The need for understanding the role of platelet function in hemostasis is of critical importance, especially in trauma hemorrhage models.

2. Materials and methods

2.1. Sample collection

Male, approximately 26-week-old Sprague Dawley rats (400–450 g) were anesthetized and catheterized. A flexible Tygon polyethylene catheter #50 (OD 0.965 mm, ID 0.58 mm, length 15 cm) was inserted into the carotid artery approximately 1.0 cm. Blood samples were collected through a 'four-way' stopcock. The catheter was kept patent with 0.25 mM sodium citrate. At the time of sample collection, 0.5 ml blood was initially collected and discarded, followed by immediate removal of 4.0 ml blood using a 5 ml syringe. Blood was immediately transferred to Vacutainer blood collection tubes (Becton Dickinson, Franklin Lakes, NJ) containing 30 USP units sodium heparin. A total of six rats were used for this study.

2.2. Immunofluorescence staining for CD3, CD16b, and CD68 cell surface receptors

All monoclonal antibodies (Mab) (Table 1) used were purchased from BD Pharmingen, San Diego, CA. Briefly, 1 ml RPMI-1640 media containing 300 mg/l

Table 1
Characteristics of monoclonal antibodies

	IL-1β	IL-2	IL-6	TNF-α	T-cells	Granulocyte and macrophage
Host species	Goat	Goat	Goat	Goat	Mouse	Mouse
Target species	Rat	Rat	Rat	Rat	Rat	Rat
Specificity	rrIL-1β	rrIL-2	rrIL-6	rrTNF-α	CD3	CD16b/CD68
Isotype	IgG	IgG	IgG	IgG	IgG1	IgG2

Specificity directed by *E. coli*-derived, recombinant rat cytokine (rrXXX) immunogen or cluster designation (CD).

L-glutamine (Life Technologies, Rockville, MD) was added to 1 ml sodium-heparinized rat blood. Blood samples comprising each treatment condition were treated with 20 μl 1.0×10^{-5} M SP (1.0×10^{-7} M final concentration). Control samples received 20 μl phosphate buffered saline containing 0.5% (w/v) bovine serum albumin (PBS-BSA). Every sample received 10 μg Brefeldin A (Sigma Chemical Company, St. Louis, MO). Control samples received 25 ng Phorbol meristate acetate (PMA, Sigma Chemical Company, St. Louis, MO) and 1 μg Ionomycin (Calbiochem, La Jolla, CA). PMA incubation time was determined by a series of time dependent experiments indicating no increase in stimulation after 4 h exposure. Exposure to PMA in excess of 4 h, e.g., 8 h resulted in noticeable cell death (data not shown). All samples were mixed gently and incubated at 37 °C in a 5% CO_2 atmosphere for 4 h. Leukocytes were stained by adding 20 μl of the appropriate Mab to 100 μl treated blood. Cells were then incubated at room temperature in the dark for 15 min. Excess unbound antibody was washed out by addition of 2 ml PBS-BSA, centrifuged at $200 \times g$ for 5 min and supernatant discarded. Goat-anti-mouse FITC labeled antibody (20 μl) was added to all reaction tubes containing mouse-anti-rat cell surface Mab, incubated at room temperature in the dark for 15 min, followed by centrifugation at $200 \times g$ and aspiration of the supernatant material. Lysing solution (2 ml, 1X FACS, BD San Jose, CA) was added and cells were incubated at room temperature in the dark for 10 min. Lysed red blood cells were removed by addition of 2 ml of PBS-BSA and centrifugation at $200 \times g$ for 5 min. Supernatant was carefully decanted and discarded. Cells were resuspended in 500 μl 2% (w/v) paraformaldehyde.

2.3. Intracellular immunofluorescence staining of TNF-α, IL-1β, IL-2, and IL-6

Cytokine markers were all biotinylated, mouse-anti-rat monoclonal antibodies. Fixative was washed out by addition of 2 ml PBS-BSA and subsequent centrifugation at $200 \times g$ for 5 min followed by removal of supernatant. FIX & PERM Reagent A (BD San Jose, CA) was added (100 μl) to each assay tube followed by incubation of cells at room temperature for 15 min. PBS-BSA (2 ml) was added followed by centrifugation at $200 \times g$ for 5 min and removal of supernatant. FIX & PERM Reagent B (BD San Jose, CA) was added (100 μl) to each assay tube. Appropriate intracellular cytokine Mab (20 μl) was added to respective assay tubes followed by incubation at room temperature in the dark for 15 min. PBS-BSA (2 ml) was added to each assay tube followed by centrifugation at $200 \times g$ for 5 min and subsequent removal of supernatant. Streptavidin Red-670 (20 μl, Gibco BRL, Rockville, MD) was added to all reaction mixtures containing biotinylated Mab. Following incubation for 15 min at room temperature, PBS (2 ml) was added to the reaction mixture, centrifuged at $200 \times g$ for 5 min, and supernatant was carefully decanted. The cell pellet was resuspended in 400 μl 1% (v/v) formaldehyde and stored in the dark at 4 °C prior to flow cytometric analysis.

2.4. Flow cytometry: controls, acquisition, and analysis

For all staining, the proper isotypic (IgG1, IgG2a, IgG2b, IgM) negative (background auto-fluorescence and non-specific secondary antibody binding) and positive (stimulation) controls were performed for each sample stained. All antibodies were titrated and optimal fluorescence (defined as the lowest concentration of antibody that generated the highest fluorescent intensity) determined. Cells were subjected to analysis using a FACSCalibur flow cytometer supported by CELLQuest software (Becton Dickinson, San Jose, CA). Calibration of the flow cytometer was achieved by linearity, sensitivity, compensation, and photo multiplier tube (PMT) measurement and adjustment. No instrument drift was observed since all stains were evaluated with identical instrument settings on multiple days. All values constitute the mean value of 10,000 events per sample.

2.5. Statistical analysis

One way analysis of variance (ANOVA) was employed and differences were considered significant at $p \leqslant 0.05$. Results are presented as respective means ± one SD. All statistical analyses were performed using SPSS software (Chicago, IL).

3. Results

3.1. Leukocyte sub-populations responsiveness to PMA

All experiments were conducted using blood treated under three specific conditions. Whole blood leukocytes were stained under unstimulated, PMA stimulated, and SP treatment regimens. PMA stimulation serves as a positive control demonstrating cell responsiveness and also represents any stimulation arising at the time of blood collection by comparing the cytokine production levels between unstimulated and PMA stimulation. T-cell TNF basal level expression (percent T-cells expressing) was observed to be 3.33 ± 0.75, increasing to 52.73 ± 4.83 following PMA activation ($p = 0.0025$). Basal IL-6, IL-2, and IL-1T-cell levels were 5.28 ± 1.51, 8.92 ± 0.77 and 1.83 ± 0.65, respectively. All T-cells were responsive to PMA activation increasing the percent of cells positive for cytokine expression. PMA activated values were 84.59 ± 10.57 for IL-6, 83.62 ± 8.09 for IL-2, and 35.19 ± 6.47 for IL-1 ($p < 0.05$), respectively. Likewise, the granulocyte/macrophage (PMN/MAC) population exhibited a similar response to PMA. Control TNF, IL-6, IL-2, and IL-1 levels were 2.90 ± 0.85, 10.85 ± 3.54, 4.34 ± 0.67 and 1.63 ± 0.71 while the PMA increase was 34.52 ± 6.30, 80.86 ± 7.45, 16.39 ± 4.81 and 17.36 ± 3.32, respectively ($p < 0.05$). These data are summarized in Fig. 1.

While the percent of cells expressing a particular cytokine represents measurement of the responsiveness of the cell population, the mean fluorescence of each cytokine is a measure of how much cytokine is being synthesized by a given population. For the T-cell population, mean basal fluorescence intensity for TNF, IL-6, IL-2, and IL-1 was 350.96 ± 37.10, 321.65 ± 35.71, 398.42 ± 38.47 and 649.95 ± 91.72, respectively. The PMA activated mean fluorescence intensity was 976.06 ± 81.77, 530.46 ± 38.01, 228.82 ± 25.66 and 601.86 ± 31.30, respectively. These data demonstrate that the T-cell population synthesizes primarily TNF and IL-6 ($p < 0.05$) while there is no significant increase in the amount of IL-2 and IL-1 produced following PMA stimulation. These data are summarized in Fig. 2.

In contrast, production of cytokines by the PMN/MAC population exhibited a more robust expression than that observed for T-cells. PMN/MAC basal levels of TNF, IL-6, IL-2, and IL-1 were 409.37 ± 82.10, 293.75 ± 50.68, 495.88 ± 86.36 and 444.53 ± 80.57, respectively. Following PMA activation, these observed levels were 2792.75 ± 216.59, 2039.12 ± 117.59, 853.96 ± 61.44 and 2305.47 ± 57.61, respectively ($p < 0.05$). These data are summarized in Fig. 2.

3.2. T-cell response to SP

CD3 receptor (T-cell) staining was used to gate the T-cell population. The percent unstimulated T-cells expressing TNF, IL-6, IL-2, and IL-1 was 3.33 ± 0.75, 5.28 ± 1.51, 8.92 ± 0.77 and 1.83 ± 0.65, respectively. The addition of SP increased the percent T-cells expressing TNF, IL-6, IL-2, and IL-1 to 45.99 ± 4.57, 52.01 ± 3.49, 46.60 ± 1.34 and 20.52 ± 4.03, respectively ($p < 0.05$). These data are summarized in Fig. 3.

As shown in Fig. 4, control mean fluorescence intensity values for TNF, IL-6, IL-2, and IL-1 expression in T-cells was 350.96 ± 37.10, 321.65 ± 35.71, 398.42 ± 38.47 and 649.95 ± 91.72, respectively. Following treatment with SP, the observed mean fluorescence intensity values were 600.06 ± 10.30, 256.76 ± 35.72, 208.34 ± 33.73 and 308.92 ± 29.56, respectively.

3.3. Granulocyte/macrophage response to SP

Using a fluorescent antibody against CD16b/CD68, we gated the PMN/MAC population for independent analysis. The percent unstimulated PMN/MAC

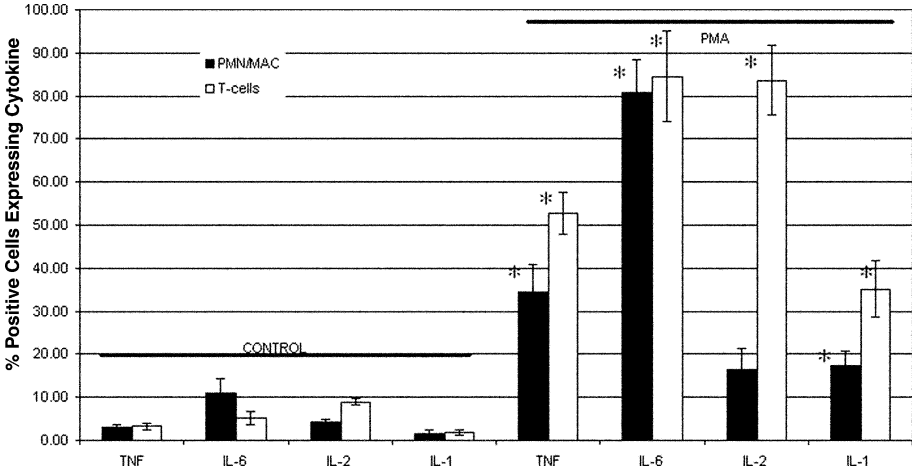

Fig. 1. Response of T-cell (open bars) and neutrophil/macrophage (closed bars) leukocyte subpopulations to PMA (250 ng/ml). The *y*-axis represents the subpopulation percent positive cells expressing cytokine. Each point represents the mean value ($n = 6$) ±SEM, $p < 0.05$.

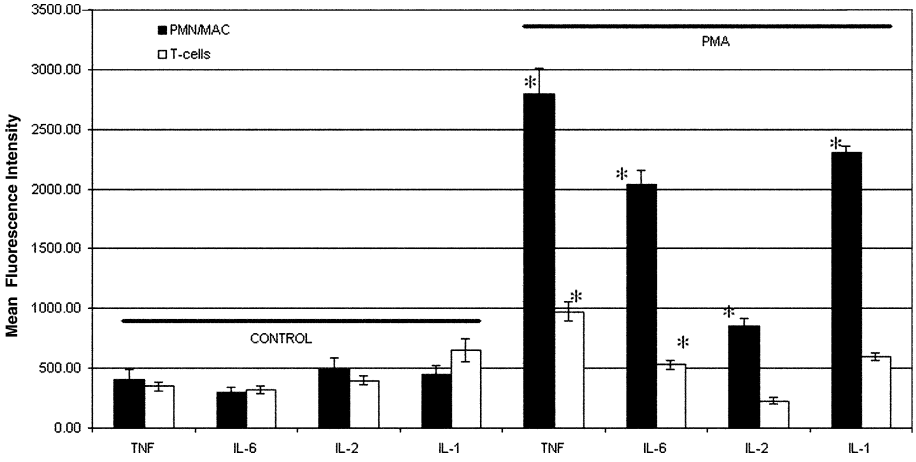

Fig. 2. Response of T-cell (open bars) and neutrophil/macrophage (closed bars) leukocyte subpopulations to PMA (250 ng/ml). The *y*-axis represents the total amount of cytokine expressed by each leukocyte subpopulation. Each point represents the mean value ($n = 6$) ±SEM, $p < 0.05$.

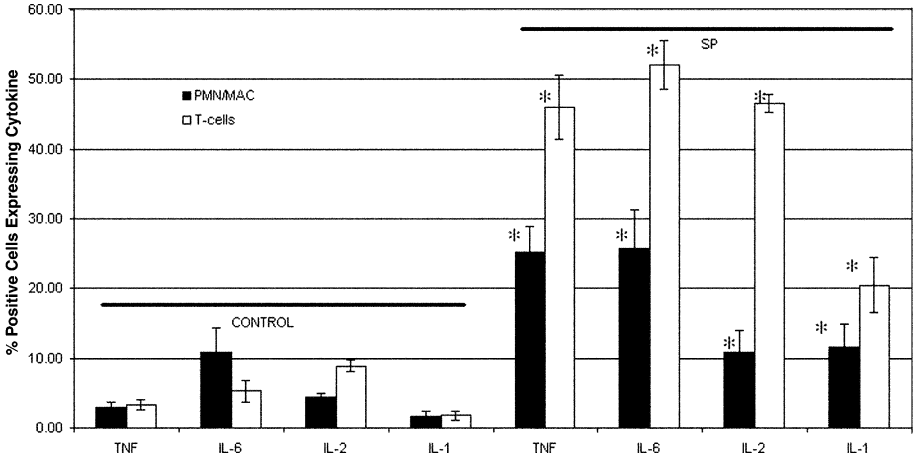

Fig. 3. Response of T-cell (open bars) and neutrophil/macrophage (closed bars) leukocyte subpopulations to SP (1.0×10^{-5} M). The *y*-axis represents the subpopulation percent positive cells expressing cytokine. Each point represents the mean value ($n = 6$) ±SEM, $p < 0.05$.

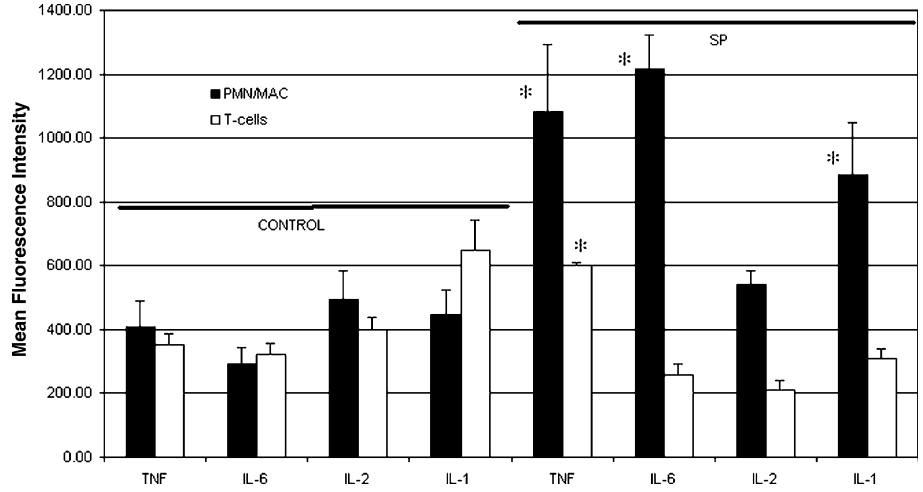

Fig. 4. Response of T-cell (open bars) and neutrophil/macrophage (closed bars) leukocyte subpopulations to SP (1.0×10^{-5} M). The *y*-axis represents the total amount of cytokine expressed by each leukocyte subpopulation. Each point represents the mean value ($n = 6$) ±SEM, $p < 0.05$.

expressing TNF, IL-6, IL-2, and IL-1 was 2.90 ± 0.85, 10.85 ± 3.54, 4.34 ± 0.67 and 1.63 ± 0.71, respectively. However, SP treatment increased the percentage of PMN/MAC expressing TNF, IL-6, IL-2, and IL-1 to 25.16 ± 3.80, 25.84 ± 5.47, 10.79 ± 3.28 and 11.52 ± 3.45, respectively ($p < 0.05$). These data are summarized in Fig. 3.

Control mean fluorescence intensity values for TNF, IL-6, IL-2, and IL-1 expression in the PMN/MAC population were 409.37 ± 82.19, 293.75 ± 50.68, 495.88 ± 86.36 and 444.53 ± 80.57, respectively. As indicated in Fig. 4, addition of SP resulted in mean fluorescence intensity values of 1080.55 ± 213.43, 1214.61 ± 109.18, 541.97 ± 42.76 and 883.64 ± 165.40, respectively ($p < 0.05$). Pretreatment with 10^{-6} M Spantide II inhibited SP mediated cytokine production and expression in both granulocyte and macrophage subpopulations is consistent with SP mediated effects via tachykinin receptors, e.g., NK1 (data not shown).

4. Discussion

Previous studies have documented the secretion of cytokines by various cell types as direct stimulation of the NK1 receptor by SP. Levite demonstrated that SP induced a marked secretion of IL-2, IL-4, and IL-10 from T helper cells (Levite, 1998). Concurrently, Kawamura and coworkers presented evidence that mouse T-cell subsets secreted INF-γ and IL-4 following treatment with SP (Kawamura et al., 1998). Likewise, Veronesi and coworkers demonstrated SP stimulated release of IL-6, IL-8, and TNF-α in human bronchial epithelial cells (Veronesi et al., 1999). It has been demonstrated that the SP induced cytokine production is a direct effect induced via the SP (NK1) receptor. At minimum, these findings suggest that the nervous system may in some way control the immunological response, at localized areas, after injury or during the inflammation process. There are extensive reports indicating SP at concentrations between 10^{-5} and 10^{-8} M are effective in inducing stimulating responses in immune cells (Rameshwar et al., 1992; Tanaka et al., 1988; Payan, 1985; Weidner et al., 2000; Kito et al., 1986; Petersen et al., 1997). More importantly, pathophysiological concentrations of SP have been detected in the 10^{-6}–10^{-10} M range using microdialysis and HPLC (Bowles et al., 2003). Based on these data an effective SP concentration of 10^{-7} was used for this study.

Of significance, the study of cytokine secretion has traditionally entailed culturing of cells of interest and analysis of decanted supernatants for the presence of cytokines. This approach presents a distinct disadvantage because secretion cannot be specifically associated with the secreting cell(s). Our study employs the use of a two-color, flow cytometric immunolabeling technique resulting in (1) detection of the secreted cytokine as well as (2) identification of the cell, i.e., sub-population producing the cytokine of interest.

Earlier studies have been traditionally conducted in a mouse model due to lack of availability of antibodies against rat proteins (i.e. Cytokines). Published data demonstrate that SP production and its effect on immune cells is valid using multiple species. Recent data corroborate findings indicating rat cytokine production to be mediated by SP (Cochiara et al., 1999; Sauerstein et al., 2000). Small differences have been demonstrated between human and rat systems as to effective concentrations as well as intensity of effect, but overall the effect of SP is consistent through species. This comes as no surprise since the amino acid sequence of mammalian as well as invertebrate tachykinins has been conserved throughout evolution (Cuello, 1987).

TNF-α, a potent lymphoid factor that exerts cytotoxic effects on a wide range of tumor cells and other target cells, appears to be upregulated upon activation by SP in both T-cell and PMN/MAC populations. SP increases the response of IL-6 secreting cells in both populations tested while the overall synthesis of IL-6 was increased in the PMN/MAC population while no significant change was observed in the T-cell population. The effect on SP on IL-2 secretion was similar to that of IL-6. Both T-cell and PMN/MAC populations exhibited an increase in the percent of cells expressing IL-2 while there was no significant change in the total synthesis of this cytokine. The effect on IL-1β was more like TNF-α. The percent of both PMN/MAC and T-cells was increased upon exposure to SP. Overall, the synthesis of IL-1β was significantly increased in the PMN/MAC population while the T-cell population appeared not to be affected by SP.

Our findings indicate that SP has an inductive effect, i.e., increasing the number of T-cells and granulocyte/macrophage populations producing cytokine as well as an overall increase in the total amount cytokine synthesized. The PMN/MAC population demonstrated a higher level of cytokine synthesized while the T-cell population exhibited the highest percentage of cells producing cytokine. This is due to the total number of cells involved. For example, in normal rats, the percentage of T-cells in whole blood is approximately 25% in a blood sample comprised of a white blood cell (WBC) count of 4.0×10^6 per ml. This translates to approximately 1.0×10^6 T-cells/ml. Likewise; approximately 63% granulocyte/macrophages comprises rat whole blood corresponding to approximately 2.5×10^6 PMN/MAC cells/ml. The percentage of SP treated T-cells expressing TNF-α was 45.99% or approximately 4.6×10^5 cells, while the corresponding percentage of PMN/MAC cells was 25.2% or approximately 6.3×10^5 cells. This demonstrates that even though the population percentage is higher with regards to T-cell number, the

total amount of cytokine synthesized is larger in the PMN/MAC population. The overall contribution of cytokine secretion is greater in the PMN/MAC than that observed in the T-cell population. The pattern of higher cytokine expression in the T-cell population while total cytokine synthesis is higher in the PMN/MAC population is consistent throughout this study.

This observation is consistent with that of an inflammation theme. During inflammation, neutrophils and macrophages are the two primary cell types that infiltrate the wound in the early stages of injury or infection. Concurrent with this cellular infiltration is robust cytokine signaling required for resisting infection and/or amelioration of tissue injury. Subsequently, T-cells infiltrate the wound affording for a more specialized function of significantly longer duration. Teleologically, this is consistent with reduced T-cell population cytokine secretion but all cells comprising the population (i.e. activated cells) would express the proper signal mechanism. Furthermore, data reported here are consistent with events required for injury or infection management during inflammation.

Results from our study as well as data derived from various laboratories at minimum suggest the inflammation cascade to be mediated via cytokines and that infiltrating leukocytes play a major role in the localized (tissue) inflammation process. Furthermore, SP is found in abundance in such inflamed environments. Thus, there is strong suspicion that SP and cytokine production by leukocyte subpopulations reside at the forefront of inflammation and trauma. Although speculative, extrapolation of our whole blood (in vitro) findings to that of in vivo events during wound healing and inflammation is consistent with the above theme of contribution of cytokine synthesis at the site of injury to arise to an even greater extent from cells that primarily infiltrate the wound after trauma or inflammation, i.e., granulocytes and macrophages.

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